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Torino, July 5, 2004

Applicant's Case E-1712/03

Attention: Dr. A. Weijland – Examiner

Re: International patent Application No. PCT/EP03/05993 in the name of IGEA S.r.l.

Dear Sirs,

Reference is made to the first written opinion dated 02.04.2004 relating to the above case.

In reply to the above written opinion, the applicant files, for the purposes of PCT preliminary examination, amended claims 1 to 14 to substitute pending claims 1 to 16.

With more detail, amended claim 1 contains the features of original claims 1 and 12. Original claim 12 has been deleted as now redundant.

Original independent claim 13 has been cancelled only for the purposes of International Examination.

Original claims 14 to 16 have been renumbered as claims 12 to 14. The tem "mathematical combination" has been substituted with the limiting term "the ratio" through all the claims.

Art 33(2) PCT.

The applicant is of the opinion that the invention is new and original with respect to cited prior art.

To that regard, our observations are the following:

Document **D1** (that is a previous application filed by the same applicant) describes a an electro-poration method of a substrate (see figure 2) wherein:

- The impedance $Z(\omega) = V/I$ (wherein V represent voltage and I current) of the substrate is determined;
- if the impedance $Z(\omega)$ is too much low (block 110) no action is performed;
- if the impedance $Z(\omega)$ is acceptable (block 110) an objective voltage is calculated whose amplitude depends on the measured impendence $Z(\omega)$; and
- pulses (130) are generated whose amplitude correspond to the determined voltage.

Document **D1** does not describe any control for applying a stimulating signal in a controlled manner according to the waveform of the initial portion of curve C_{GT} representing the ratio of current I versus voltage V; i.e. GT = I/V.

Therefore amended claim 1 has to be considered **new** with respect to document **D1**.

Moreover, no suggestion to the above claimed combination may derive from document **D1** because:

a) in document **D1** a completely different parameter is measured; i.e. the impedance that is voltage /current whilst the present invention is based on the detection of an <u>opposite solution</u> i.e. current/voltage; and

b) document **D1** does not provide any hint of the distinguishing feature of deciding which action are to be taken depending only on the <u>initial portion</u> of a curve.

It is thus believed that the claimed solution has to be considered also original with respect to document **D1**.

Similar observations apply to document **D2** wherein a measure of the impedance is performed (page 19, first paragraph) in order to detect when the electro-permabilization process begins so that the amount of current supplied is regulated (see page 20, lines 12-15) in order to avoid irreversible cells damage.

Therefore the applicant believes that also document **D2** does not describe/suggest the above a) and b) distinguishing features.

Moreover, as already pointed out by the Examiner, document **D2** contains a general statement (page 29, lines 13-15) that the measurement of a current - voltage may provide some information of the cell integrity.

This passage – however – must not be interpreted as a direct suggestion of the detection/calculation of current/voltage ratio because document D2, with this regard, is very generic and seem to refer to the described main embodiment, i.e. the measurement of the impedance, i.e. voltage/current see the same page at lines 16,17 *"Measuring the current-voltage (impedance relation across the cell membrane could also detect if the membrane was impaired by these chemicals)"*.

In addition this vague statement may not in any case suggest the specific use of initial portion of the curve C_{GT} as presently claimed.

Similar observation also apply to documents **D3** and **D4**.

Reconsideration of the previous negative assessment to the validity of the claims it is therefore hoped.

Yours faithfully

Dr. Ing. Simone Bongiovanni

CLAIMS

1.- Electroporation device for the permeabilization of cells (C) contained in a substrate (12) comprising signal generating means (3) for generating a stimulating signal (S(t)) applied by means of electrodes (6,7) to the substrate (12) wherein an electric field (E(t)) permeabilizing the cells membranes is induced;

the device being **characterized by comprising:**

- means for measuring, calculating and monitoring (15,16,23) the instantaneous value of ~~a mathematical combination~~ the ratio (GT) of current (**ie**) flowing between said electrodes (6,7) and through the substrate (12) and the voltage (**Vp**) of the stimulating signal (S(t)) applied to the substrate (12) by means of said electrodes (6,7);

said device further comprising controlling means (100-170) for applying the stimulating signal in a controlled manner according to the waveform of an initial portion of the curve C_{GT} representing the ~~value of the mathematical combination~~ ratio (GT) in successive instants after the beginning of the application of the stimulating signal (S(t)).

2.- Device as claimed in claim 1, wherein said controlling means (100-170) comprise timing means (110) for applying said stimulating signal for a predetermined

period of time Td and analysing the initial portion of the waveform of curve C_{GT} to detect a minimum value of the curve C_{GT} within the interval t = 0 and t = Td.

3.- Device as claimed in claim 1 or 2, wherein said controlling means (100-170) calculate the slope of the waveform of curve C_{GT} after that a minimum in curve C_{GT} has been reached.

4.- Device as claimed in any of the preceding claims, wherein said controlling means (100-170) comprise hazard detecting means (120) determining the instantaneous gradient (**dG**) of said ~~mathematical combination~~ ratio (GT) after a minimum has been reached in said curve C_{GT};

said controlling means further comprise first comparing means (130) for comparing the calculated instantaneous gradient **dG** with at least a reference value (dG_{ref1}) and selecting correcting means (140,145) for performing an urgent correction to the stimulating signal S(t) in order to avoid lesions, damages or irreversible alterations in said substrate (12).

5.- Device as claimed in claim 4, wherein said correcting means (140,145) decreases the voltage of the stimulating signal S(t) in order to prevent deterioration in the cells (C).

6.- Device as claimed in any of the preceding claims, wherein said controlling means (100-170)

comprise slope determining means (150) calculating the average variation **ΔG** of said ~~mathematical combination~~ ratio **(GT)** in a time interval that is successive to the instant **Tm** wherein a minimum in the curve (C_{GT}) has been reached and that has a pre-determined time width;

said controlling means further comprising second comparing means (160) comparing the calculated average variation **ΔG** of said ~~mathematical combination~~ ratio **(GT)** with a reference interval of **ΔG** values.

7.- Device as claimed in claim 6, wherein said second comparing means (160) performs the following functions:

- if the calculated average variation **ΔG** of said ~~mathematical combination~~ ratio **(GT)** falls within the reference interval ($0 < \Delta G < \Delta G_{obb}$) continuing means (170) are selected;
- if the calculated average variation **ΔG** of said ~~mathematical combination~~ ratio **(GT)** falls outside the reference interval and it is smaller than both limits delimiting the interval ($\Delta G < 0 < \Delta G_{obb}$) adjusting means (180) are selected; and
- if the calculated average variation **ΔG** of said ~~mathematical combination~~ ratio **(GT)** falls outside the reference interval and it is greater than both limits delimiting the interval ($\Delta G > \Delta G_{obb} > 0$) correcting means (140) are selected.

8.- Device as claimed in claim 7, wherein said adjusting means (180) increase the voltage of the stimulating signal in order to increase the value of the electric field E(t) applied to the substrate (12); said adjusting means (180) subsequently selecting said means for calculating and monitoring (15,16,23) the instantaneous value of the said ratio (GT) and said controlling means.

9.- Device as claimed in claim 7, wherein said continuing means (170) increase the voltage of the stimulating signal to an objective voltage V_{opt} in order to increase the value of the electric field E(t) applied to the substrate (12) so that the value of said average variation ΔG tends to an expected value ΔG_{obb}.

10. - Device as claimed in any of the preceding claims, wherein said controlling means (100-170) detects (125) a minimum of said initial portion of said curve and determining (126) the time Tm at which the minimum is reached.

11.- Device as claimed in claim 10, wherein third comparing means (127) are provided to compare the detected time Tm with threshold values Ttmin and Ttmax;

said third comparing means (127) performing the following operations:

- if the detected Tm occurs before Ttmin (Tm < Ttmin) then correcting means (140) are selected;

- if the detected Tm occurs after Ttmax (Tm > Ttmax), then adjusting means (180) are selected; and

- - if the detected Tm occurs between Ttmin and Ttmax, then continuing means (170) are selected.

~~12. Device as claimed in any of the proceeding~~ claims, wherein said mathematical ~~combi~~nation comprise the ratio GT ~~between~~ said current (ie) and said voltage ~~(vp).~~

~~13. Electroporation device for the~~ permeabilization of cells (C) contained in a substrate (12) comprising signal generating means (3) for generating a stimulating signal (S(t)) applied by means of electrodes (6,7) to the substrate (12) wherein an electric field (E(t)) permeabilizing the cells membranes is induced;

the device being **characterized by comprising:**

- means for measuring, calculating and monitoring (15,16,23) the instantaneous value of the current **(ie)** flowing between said electrodes (6,7) and through the substrate (12);

said device further comprising controlling means (100-170) for applying the stimulating signal in a controlled manner according to the waveform of an initial portion of the curve representing the value of the current (ie) in successive instants after the

~~beginning of the application of the stimulating signal (S(t)).~~

12
~~14~~.- Use of a device as described in any of the preceding claims, to extract molecules from the living cells comprised in the substrate.

13
~~15~~.- Use of a device as described in any of the preceding claims, to introduce molecules into living cells.

14 13
~~16~~.- Use of the device as claimed in claim ~~15~~, wherein said molecules comprise one of the following:

- a DNA or a RNA molecule containing regulatory sequences and sequence coding for therapeutic genes or genes of interest for biomedical or biotechnological purposes;
- an oligonucleotide, (ribo- or deoxyribo-nucleotide, single or double strand, including the SiRNA), whether natural (phosphodiesters) or modified (inside the backbone of the oligonucleotide, such as phosphosulfates, or at the extremities, by addition of groups to protect the oligonucleotides from digestion of nucleases;
- a protein or peptide, whether natural or genetically or chemically modified, extracted from natural sources or obtained by synthesis, or a molecule simulating the structure of a

protein or peptide, whatever its structure;

- a cytotoxic agent, in particular, the antibiotic bleomycin or the cisplatinum;
- a penicillin; and

other pharmacological agents.